Science of the Total Environment 615 (2018) 208–218

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides

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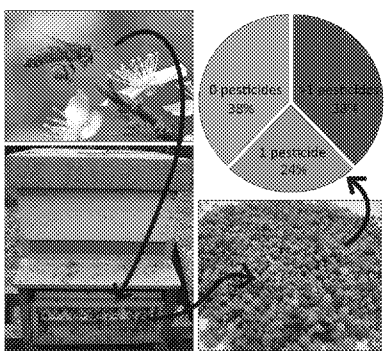
HIGHLIGHTS

- The majority (62%) of pollen samples contained at least one pesticide (2012–2014).
- Multiresidual samples (38%) were more frequent than single contaminations (24%).
- Chlorpyrifos was the most frequently detected pesticide (30%).
- Imidacloprid-contaminated samples had the highest HQ, with 12% of samples >1000.
- Health safety levels (ARfD, ADI, MRL) were exceeded in 39% of the residues.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 August 2017
Received in revised form 20 September 2017
Accepted 21 September 2017
Available online xxxx

Editor: Jay Gan

Keywords:
Bee health
Monitoring
Multiple residues
Chemical mixture
Chlorpyrifos
Imidacloprid

ABSTRACT

Honey bee (*Apis mellifera* L.) health is compromised by complex interactions between multiple stressors, among which pesticides play a major role. To better understand the extent of honey bee colonies' exposure to pesticides in time and space, we conducted a survey by collecting corbicular pollen from returning honey bee foragers in 53 Italian apiaries during the active beekeeping season of 3 subsequent years (2012–2014).

Of 554 pollen samples analysed for pesticide residues, 62% contained at least one pesticide. The overall rate of multiresidual samples (38%) was higher than the rate of single pesticide samples (24%), reaching a maximum of 7 pesticides per sample (1%). Over 3 years, 18 different pesticides were detected (10 fungicides and 8 insecticides) out of 66 analysed. Pesticide concentrations reached the level of concern for bee health (Hazard Quotient (HQ) higher than 1000) at least once in 13% of the apiaries and exceeded the thresholds of safety for human dietary intake (Acute Reference Dose (ARfD), the Acceptable Daily Intake (ADI), and the Maximum Residue Limit (MRL)) in 39% of the analysis. The pesticide which was most frequently detected was the insecticide chlorpyrifos (30% of the samples overall, exceeding ARfD, ADI, or MRL in 99% of the positive ones), followed by the fungicides mandipropamid (19%), metalaxyl (16%), spiroxamine (15%), and the neonicotinoid insecticide imidacloprid (12%). Imidacloprid had also the highest HQ level (5054, with 12% of its positive samples with HQ higher than 1000).

This 3 year survey provides further insights on the contamination caused by agricultural pesticide use on honey bee colonies. Bee-collected pollen is shown to be a valuable tool for environmental monitoring, and for the detection of illegal uses of pesticides.

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https://doi.org/10.1016/j.scitotenv.2017.09.226
0048-9697/

1. Introduction

In the last century agriculture has expanded and intensified (Ramankutty and Foley, 1999), providing higher crop yields for a growing world population. The increased agricultural practices however have had a high environmental cost: habitat loss and widespread use of pesticides have posed significant negative consequences for wild flora and fauna (Matson et al., 1997; McLaughlin and Mineau, 1995; Van Dijk et al., 2013). Thus, it is not surprising that there is an ongoing global decline of pollinators (Biesmeijer et al., 2006; Potts et al., 2010), which is alarming due to the important role pollinators play in ecological systems and crop productivity (Aizen et al., 2009; Fontaine et al., 2005; Garibaldi et al., 2011; Klein et al., 2007). Honey bees, important crop pollinators, can be considered as indicators of the health status of pollinating insects. In fact, because beekeepers rear and monitor bee colonies worldwide, they are immediately aware of changes in colony health, productivity, and behaviour. Indeed, it was beekeepers who alerted the media and scientific community about an increase in the normal rate of colony mortality around 2006 (Cox-Foster et al., 2007). The phenomenon was named Colony Collapse Disorder (CCD) or more generically "colony losses", and it engendered research initiatives across the world (Carreck and Neumann, 2010).

Various stressors have been investigated and found to be possible cause of the phenomenon: parasites and pathogens (Cornman et al., 2012; Cox-Foster et al., 2007; Dainat et al., 2012; Higes et al., 2010; Le Conte et al., 2010; Ravoet et al., 2013), pesticides (Belzunces et al., 2012; Desneux et al., 2007; Sandrock et al., 2014), climate change (Memmott et al., 2007) and nutrition (Archer et al., 2014). Much of the evidence collected in recent years suggests that a combination of these factors, acting in synchrony and with complex interactions, is responsible for the increased honeybee colony mortality. Pesticides are considered to be a key factor, as a multitude of studies have demonstrated their detrimental effects at both individual and colony level (Goulson, 2013; Sánchez-Bayo et al., 2016; Sgolastra et al., 2017b; Tosi et al., 2017; van der Sluijs et al., 2013). Many of these studies were conducted in vitro and/or in semi-field conditions and their results were questioned because of the lack of certainty about the actual pesticide exposure of bees in the field (Blacquière et al., 2012). However, recent studies have addressed the problem at the field level and have confirmed the detrimental effects of pesticide exposure for bees (Rundlöf et al., 2015; Woodcock et al., 2017). Furthermore, they have shown that a realistic scenario comprehends a continuous exposure to multiple pesticides (Botías et al., 2017; David et al., 2016; Long and Krupke, 2016). Because of the prolonged exposure to the toxins, this kind of contamination may be more harmful to honey bees than pulse exposures which are normally tested in laboratory conditions (Laycock and Cresswell, 2013).

Several of the most commonly used pesticides are systemic, protecting (and contaminating) all plant organs, including flowers—and thus nectar and pollen. Pollen is the main protein and lipid source for bee colonies and a fundamental part of the nurse bees' and larval diet (Crailsheim et al., 1992), thus its contamination results in exposure of the new generation of bees, as well as the foraging and receiver bees. Some studies already evidenced widespread contamination of pollen from agricultural landscapes, and highlighted common combinations of pesticides encountered in field environments (Bernal et al., 2010; Chauzat et al., 2006; Lambert et al., 2013; Long and Krupke, 2016; Mullin et al., 2010; Smodis Skerl et al., 2010). Advocates of chemical plant protection claim that if the products are used according to good agricultural practices the effect on the environment should be negligible (Cutler et al., 2014). However, exposure to low levels of pesticides can elicit sublethal effects on bees, not killing them outright but affecting their behaviour and immune system (Desneux et al., 2007). The detection of residues at very low levels has become possible, in recent years, as new analytical techniques have been developed (Stachniuk and Fornal, 2016).

Foraging honey bees fly to an average distance of about 1.5 km from the colony (Steffan-Dewenter and Kuhn, 2003; Visscher and Seeley, 1982), meaning that an area of approximately 7 km^2 around the hive is visited by foraging bees. The average size of a European farm is 0.16 km^2 (Eurostat, 2012), thus a foraging surface of 7 km^2 is normally covered by several crops, exposing a colony placed in a rural area to multiple pesticides used for different crops. Furthermore, a multitude of pesticides are available, for example Italian farmers have access to approximately 130 different active ingredients (aa.ii.), alone or in combination, in about 1280 commercialized products for plant protection (Ministero del lavoro della salute e delle politiche sociali, 2014).

The aim of this study was to investigate the extent of honey bee exposure to agricultural pesticide residues in managed honey producing colonies. This was achieved by analysing corbicular pollen from returning forager bees (it has been shown that pollen loads are the best matrix for assessing ongoing pesticide contamination in the environment (Chauzat et al., 2011)) and using residue levels to estimate the risk hazard for honey bees. Furthermore, as pollen is also used for human consumption as a "health food supplement" (Campos et al., 2003; Carpes et al., 2009; Graikou et al., 2011; LeBlanc et al., 2009), the obtained results were compared with regulatory agency levels of concern for acute or chronic exposure in humans.

2. Material and methods

2.1. Survey period and sites

We used 53 commercial apiary sites located in Italy (22 apiaries in 2012, 24 in 2013 and 15 in 2014; 8 apiaries were used multiple years) (Fig. 1). A total number of 554 pollen samples were collected between March and September of 3 consecutive years, from 2012 to 2014. Overall, the apiaries were located in proximity of agricultural areas and were randomly selected across Italy based on apiary size, beekeeper's experience and beekeeper's ability to adhere to the working protocol of the survey. Beekeepers experience was estimated based on years of experience, membership in a beekeeping association, and training level (frequency of beekeeping meetings, conferences, workshops, and seminars attended) (EFSA, 2016). About 65% of the beekeepers managed their apiaries according to the organic production protocols (European Council, 2007). Within each apiary, 5 queen-right and healthy (i.e. no disease symptoms) honey bee colonies (*Apis mellifera* L.) were used for pollen collection.

2.2. Pollen collection

Colony management and pollen sampling and shipment were carried out by the beekeepers and apiary technicians. They were provided with a working protocol defining all monitoring details, and were personally instructed by expert beekeepers and ecotoxicologists in ad-hoc meetings to improve the harmonization of the procedure across apiaries and beekeepers.

Commercially available pollen traps were used to dislodge the pollen pellets from the corbiculae of returning foraging bees. The pollen traps were kept in place until 100 g of pollen pellets were collected (typically 2–7 days). The sampling period and success varied in relation to weather conditions and pollen import by the colonies. Samples were collected during the active beekeeping season, in the most critical periods for pesticide contamination (e.g. concomitantly with agricultural pesticide treatments), based on expert experience (i.e. consultation of farmers, beekeepers, and agronomists) on the agricultural practices in their area. After collection, the pollen pellets were homogenized using a glass jar, and 100 g were subsampled and frozen at −20 °C. A cool-box was used for shipment of the samples from the

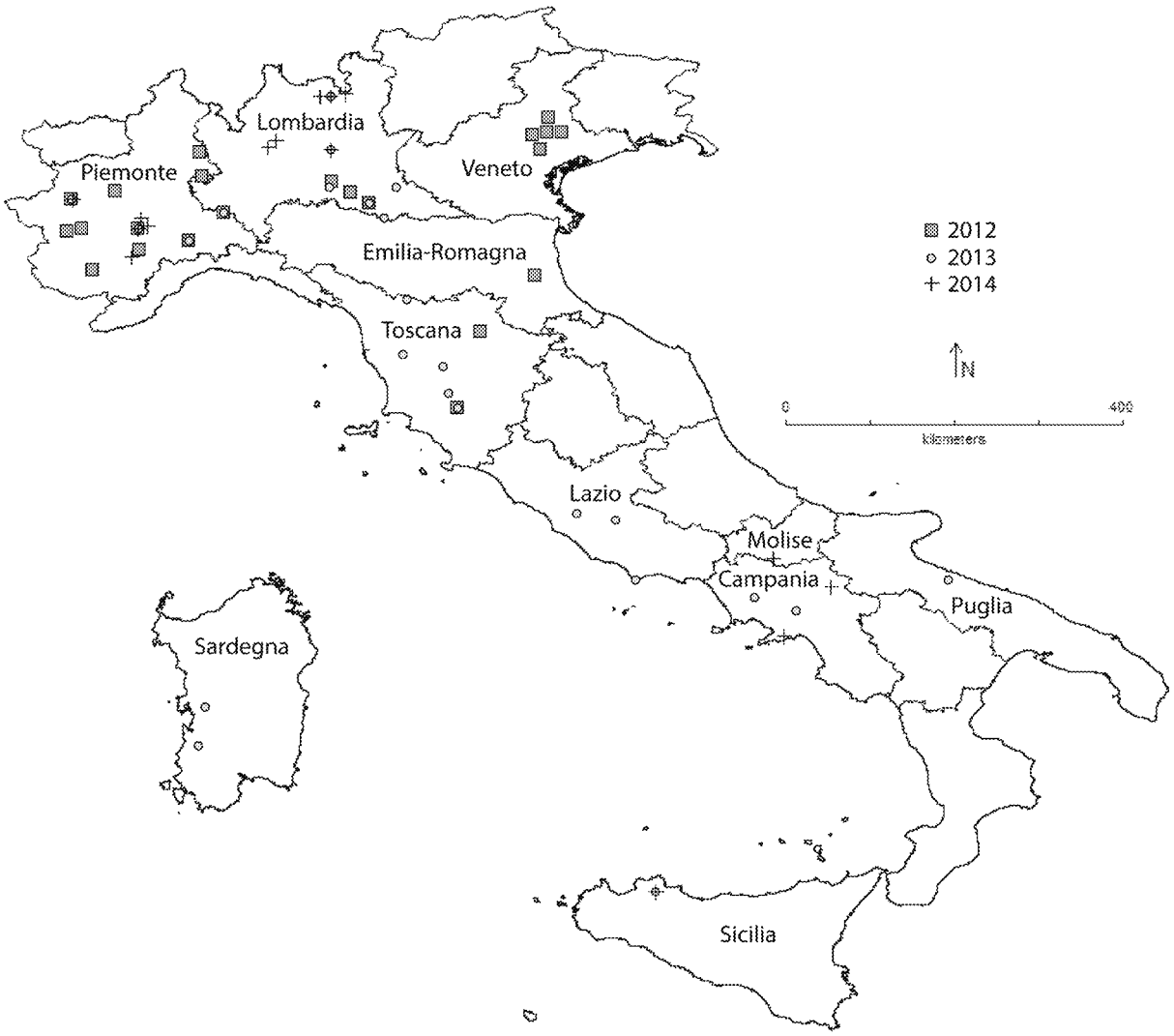

Fig. 1. Map of Italy with the locations of the apiaries used for each year of the survey. We used 22, 24 and 15 apiaries during the 2012, 2013 and 2014 seasons, respectively. The name of the regions involved is reported. To facilitate display, overlapping symbols representing different apiaries in a same year were slightly separated.

apiary to the laboratory, to ensure that samples were maintained frozen until analysis.

2.3. Pesticide residue analysis

A high sensitivity method was developed and validated for the detection of residues in honey bee-collected pollen of 66 pesticides most commonly used in Italy, including acaricides, fungicides, insecticides, nematicides, and some metabolites(Table 1).

The pollen was prepared in two steps. First, a solid/liquid extraction with solvent and MSPD purification: 10 g of each pollen sample was extracted with acetonitrile/water, followed by liquid/liquid purification with hexane and combined with MSPD purification on PSA and Salts. Then, the purified extract was concentrated to below 100 μL volume and injected into UPLC-MS/MS (Ultra Pressure Liquid Chromatography coupled with tandem mass spectrometry), programmed in MRM (Multiple Reaction Monitor) mode with two transition/molecule (Wiest et al., 2011).

Method validation was carried out according to European Union Directive 2002/657/CE (EU, 2002) at concentration level of 0.25–2.5, 5.0 ng/g depending on sensitivity of molecules. Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated (Table 1). All the collected pollen samples were analysed.

2.4. Hazard characterisation and statistical analysis

Samples with concentrations between the LOQ and the LOD were included in the calculation of the mean using the arithmetic mean of LOQ and LOD (Chauzat et al., 2006, 2011). Samples with concentrations lower than LOD were not included in the calculation of the mean (Chauzat et al., 2011).

We estimated the maximum Hazard Quotient (HQ) for honey bees for each single pesticide overall (Table 2) and for each sample across space and time (Fig. 2) using the methods described by (Stoner and Eitzer, 2013; Traynor et al., 2016). Briefly, the HQ was calculated dividing the maximum residue (μg kg^{-1}) of each pesticide by its respective oral LD_{50} (μg bee^{-1}) (EFSA, 2011). We used the LD_{50} values (Supplementary Table S1) reported by the University of Hertfordshire Pesticide Properties DataBase (Lewis et al., 2016). We estimated the HQ of pollen samples which typically contained multiple residues, assuming additive toxic effects. We excluded possible synergistic or antagonistic effects due to lack of quantitative data on interactions between most of the pesticides under assessment. Based upon the average daily pollen consumption of a nurse bee (9.5 mg/bee/day, (Crailsheim et al., 1992)), a Hazard Quotient of 1000 corresponds to consuming 1% of the median lethal dose (LD_{50}) per day, which adds up to 10% after the 10 day nursing phase (assuming no degradation or detoxification). Using a standard

Table 1
List of screened active ingredients with respective Limit of Quantification (LOQ) and Limit of Detection (LOD).

Active ingredient	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Active ingredient	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Acrinathrin	2.50	7.50	Flusilazole	0.25	2.50
Azinphos methyl	1.00	5.00	Fluvalinate	2.50	7.50
Azoxystrobin	0.25	2.50	Heptenofos	2.50	7.50
Benalaxyl	0.25	2.50	Imazalil	0.25	2.50
Bifenthrin	2.50	7.50	Imidacloprid	0.25	2.50
Bitertanol	0.25	2.50	Iprovalicarb	1.00	5.00
Boscalid	2.50	7.50	Kresoxim methyl	2.50	7.50
Buprofezin	2.50	7.50	Lambda-Cyhalothrin	2.50	7.50
Carbaryl	0.25	2.50	Linuron	2.50	7.50
Chlorfenvinphos	0.25	2.50	Malathion	1.00	5.00
Chlorpyrifos	1.00	5.00	Mandipropamid	0.25	2.50
Chlorpyrifos methyl	1.00	5.00	Metalaxyl	1.00	5.00
Chlothianidin	2.50	7.50	Mevinphos	1.00	5.00
Coumaphos	1.00	5.00	Nuarimol	1.00	5.00
Cyazofamid	2.50	7.50	Oxadixyl	1.00	5.00
Cyfluthrin	1.00	5.00	Parathon	2.50	7.50
Diazinon	1.00	5.00	Parathon methyl	2.50	7.50
Dichlorvos	2.50	7.50	Phenthoate	2.50	7.50
Dichlofluanid	1.00	5.00	Phosmet	0.25	2.50
Diethofencarb	0.25	2.50	Pirimicarb	1.00	5.00
Difenoconazole	0.25	2.50	Pirimiphos	1.00	5.00
Dimethoate	0.25	2.50	Pirimiphos methyl	1.00	5.00
Esfenvalerate	2.50	7.50	Propargite	0.25	2.50
Ethion	2.50	7.50	Propyzamide	1.00	5.00
Etofenprox	2.50	7.50	Pyrazophos	0.25	2.50
Etrimfos	2.50	7.50	Quinalphos	2.50	7.50
Fenarimol	2.50	7.50	Spiroxamine	0.25	2.50
Fenazaquin	2.50	7.50	Tebuconazole	0.25	2.50
Fenbuconazole	1.00	5.00	Tebufenpyrad	1.00	5.00
Fenhexamid	0.25	2.50	Thiamethoxam	0.25	2.50
Fenitrothion	2.50	7.50	Thiabendazole	2.50	7.50
Fenthion	2.50	7.50	Tolyfluanid	2.50	7.50
Fluopicolide	3.00	10.00	Trifloxystrobin	0.25	2.50

safety factor of 1/10th of the LD_{50} (Atkins et al., 1981), the HQ value of 1000 would correspond to the threshold at which a pesticide elicits toxic effects, and is therefore considered as the limit of concern for bee health (Stoner and Eitzer, 2013; Traynor et al., 2016).

Maximum residues detected in the pollen were compared with the Acute Reference Dose (ARfD), the Acceptable Daily Intake (ADI), and the Maximum Residue Limit (MRL) in pollen (EU data on residues of vegetal pollens—honey and other apiculture products) (European Commission, 2016). ARfD is the amount of a chemical that can be consumed by a person at one meal or on one day that would lead to no harm, and ADI is the quantity of a chemical that can be consumed every day for a life-time causing no harm (on the basis of all known facts) (Renwick, 2002). MRL is the maximum concentration of pesticide residue legally permitted in or on food commodities or animal feeds (EFSA, 2017).We used JMP v.10.0 (SAS Statistical Software) for the statistical analysis, and DIVA-GIS v.7.5.0.0 (http://www.diva-gis.org/) to create the map in Fig. 1.

3. Results

Of the 554 pollen samples collected in the 3 year survey, 62% contained at least one of the screened pesticides (Table 3). The overall rate of multiresidual samples (38%) was higher than the rate of single pesticide samples (24%), and up to 7 pesticides per sample were found (two samples in 2012, one in 2013; Table 3).

Pesticides contaminated the samples in all months and years, except September 2013 ($N = 4$; Fig. 3). We found multiresidual samples in all months and years too, except September 2012 ($N = 26$), September 2013 (N = 4), and March 2014 ($N = 3$) (Fig. 3, Table 3). Overall, 18 different pesticides were detected, 10 fungicides and 8 insecticides (Table 2, Supplementary Tables S1 and S2).

Eight systemic pesticides (6 fungicides and 2 neonicotinoid insecticides: imidacloprid and thiamethoxam) contaminated 36% of the positive samples overall (Table 3).

Seven pesticides were present in all 3 years of the survey (Table 2, Supplementary Table S1). The pesticide which was most frequently detected was the insecticide chlorpyrifos (30% of the samples overall, 46% in 2014), followed by the fungicides mandipropamid (19%), metalaxyl (16%), spiroxamine (15%) and the neonicotinoid insecticide imidacloprid (12%). Of these, metalaxyl was the one with the highest mean and maximum level of residues (respectively 60 μg kg^{-1} and 2463 μg kg^{-1}, June 2012, Apiary 1 in Giavera del Montello (TR), Veneto), and with the highest overall mean value of residues exceeding the MRL. Three other pesticides had maximum residue levels over 100 μg kg^{-1}: mandipropamid (261 μg kg^{-1}, June 2012, Apiary 2 in Giavera del Montello (TR), Veneto), chlorpyrifos (179 μg kg^{-1}, July 2014, Cisterna d'Asti, Piemonte) and dimethoate (163 μg kg^{-1}, same sample that contained the highest level of metalaxyl: June 2012, Apiary 1 in Giavera del Montello). We observed a seasonal effect, with slightly higher rates of positive samples in the summer months (Table 3, Fig. 3). However, some apiaries were consistently contaminated by pesticides throughout the season, and over the years (for example in Cisterna d'Asti in Piemonte, and Ponte in Valtellina in Lombardia, Fig. 2).

In 8 of the 11 regions in which the sampling took place, more than half of the collected samples contained residues of one or more pesticides (Supplementary Fig. S1). Emilia-Romagna ($N = 10$) and Puglia ($N = 19$) had 100% of positive samples, with chlorpyrifos present in all samples in E-R, and all but one in Puglia. The regions with the highest number of samples collected showed a consistently high proportion of positive residues (Veneto: $N = 105$, 60% positive samples; Piemonte: $N = 170$, 59%; Lombardia: $N = 123$, 72%).

The Hazard Quotient was higher than 500 in 9% of the positive samples, and 3% had an HQ higher than 1000 (Fig. 2). The

Table 2
Residue levels of the pesticides detected in the 3-year survey. The type of each a.i. is reported (I = insecticide, F = fungicide, S = systemic, A = acaricide), as well as the major crops it is used on, the authorised countries, and the EU ARfD (Acute Reference Dose), ADI (Acceptable Daily Intake), and MRL (Maximum Residue Limit) levels. For each pesticide, we report the total number of registered products (n), the proportion of positive samples (%), mean (μg kg^{-1}), median (μg kg^{-1}), standard deviation (μg kg^{-1}), maximum (μg kg^{-1}) and minimum (μg kg^{-1}) concentrations, the total number of samples exceeding ARfD, ADI or MRL, and the maximum Hazard Quotient (HQ). Three pesticides were not authorised for use (pesticide name followed by "†"). Seven pesticides were detected in all years (pesticide name followed by "*"). $N = 554$ pollen samples. The residues detected in each of the 3 years of the survey is reported in Supplementary Table S1, and Supplementary Table S2 reports more details on the use and registration of these pesticides.

Active ingredient	A.i. type	Authorised countries (N)	Registered products (N)	ARfD (μg kg^{-1} bw)	ADI (μg kg^{-1} bw/day)	MRL (μg kg^{-1})	2012–2014 (554)										
							Positive (%)	Mean (μg kg^{-1})	Median (μg kg^{-1})	St Dev	Min (μg kg^{-1})	Max (μg kg^{-1})	Exceeding ARfD (N)	Exceeding ADI (N)	Exceeding MRL (N)	LD_{50} 48 h (ng/bee)	Max HQ
Azoxystrobin	F	27	8	N/A	200	50	2.9	8	1	13	1	54	N/A	0	1	>25,000	2
Benalaxyl	F S	18	35	N/A	40	N/A	7.8	5	1	9	1	45	N/A	1	N/A	>100,000	<1
Boscalid	F	27	3	N/A	40	500	0.7	23	14	25	5	58	N/A	1	0	100,000	<1
Carbaryl†	I	0	0	10	7.5	50	0.2	1	1		1	1	0	0	0	210	2
Chlorfenvinphos†	I A	0	0	N/A	0.5	10	4.5	5	1	9	1	39	N/A	12	4	550	72
Chlorpyrifos*	I	21	115	5	1	50	30.3	10	5	17	0	179	87	166	4	250	718
Dimethoate*	I	23	104	10	1	10	7.9	7	1	25	0	163	3	23	3	100	1633
Fenazaquin	I A	8	2	100	5	10	0.4	0	0	0	0	0	0	0	0	4290	<1
Fluopicolide	F S	26	2	180	80	50	2.3	5	0	10	0	34	0	0	0	>241,000	<1
Imidacloprid	I S	28	52	80	60	50	12.5	2	1	3	1	19	0	0	0	4	5054
Iprovalicarb	F S	15	3	N/A	15	50	1.1	4	3	2	3	8	N/A	0	0	>199,000	<1
Mandipropamid*	F	28	1	N/A	150	50	19.5	9	1	28	1	261	N/A	1	3	>200,000	<1
Metalaxyl*	F S	9	9	500	80	50	15.9	60	3	300	0	2463	3	5	7	269,000	9
Phenthoate†	I	0	0	N/A	3	N/A	3.1	1	0	2	0	5	N/A	1	N/A	306	11
Spiroxamine*	F S	23	1	100	25	N/A	15.0	2	1	3	0	18	0	0	N/A	>100,000	<1
Tebuconazole	F S	27	90	30	30	50	10.8	6	3	10	1	52	3	3	1	>83,050	<1
Thiamethoxam*	I S	25	9	500	26	10	4.5	1	1	0	0	3	0	0	0	5	548
Trifloxystrobin	F	24	1	N/A	100	50	5.6	3	1	8	0	46	N/A	0	0	>110,000	<1

insecticides imidacloprid (HQ = 5054), dimethoate (HQ = 1633), chlorpyrifos (HQ = 718), and thiamethoxam (HQ = 548) had the highest HQ values (Table 2). More specifically, 12% of the imidacloprid-contaminated samples had a HQ higher than 1000 (4% in 2012, 4% in 2013, and 3% in 2014). The highest HQ levels were found in Andria (BT, Puglia) in 2013 (HQ = 5054, with 19 μg kg^{-1} of imidacloprid), and in Pelago (FI, Toscana) in 2012 (HQ = 4595, with 17 μg kg^{-1} of imidacloprid). The nurse bees living in these two apiaries were exposed to pesticide levels up to 5% of the LD_{50} of imidacloprid per day, corresponding to a 50% mortality dose over 10 days. Over 3 years, two apiaries were exposed to HQ levels higher than 1000 twice in a year: Castelnuovo Scrivia (AL, Piemonte, Northern Italy) in July 2013 and Villa di Tirano (SO, Lombardia, Northern Italy) in April and May 2014.

During the 3 years of survey, the 39% of the residues exceeded the EU safety and legal levels of pesticide in pollen (ARfD, ADI, and MRL, Table 2), with ten pesticides (azoxystrobin, benalaxyl, boscalid, chlorfenvinphos, chlorpyrifos, dimethoate, mandipropamid, metalaxyl, phenthoate, tebuconazole) involved. The pesticide that mostly contributed to these results was chlorpyrifos, with 166 samples (out of 168 chlorpyrifos positive, 99%) above ADI (1 μg kg^{-1}; 34 in 2012, 58 in 2013, 74 in 2014), 87 samples above ARfD (5 μg kg^{-1}, 52%), and 4 samples above ADI, ARfD, and MRL (50 μg kg^{-1}, 2%) (Table 2).

This study shows that banned pesticides are still illegally used. We detected an organophosphate insecticide banned in Italy since 2003 (phentoate, 17 positive samples in Piemonte (4), Veneto (7), Emilia-Romagna (3), Toscana (3), in 2012, from a total of 8 apiaries, Supplementary Table S2) a carbamate insecticide banned since 2007 (carbaryl, 1 positive sample in Piemonte, in 2012) and an organophosphate insecticide banned since 2006 in the EU (2006) and with no registered products in Italy since 2003 (chlorfenvinphos, 25 positive samples in Piemonte (14), Lombardia (1), Veneto (9), Emilia-Romagna (1), from a total of 11 apiaries).

4. Discussion

The active ingredient with the highest frequency of residues (30%) was chlorpyrifos, an organophosphate with contact and stomach action (acetylcholinesterase inhibitor) which has long been registered for both indoor and outdoor use, due to its broad target spectrum. According to Dow AgroSciences "Chlorpyrifos is one of the most widely used pest control products in the world. It is authorized for use in about 100 nations, including the U.S., Canada, the United Kingdom, Spain, France, Italy, Japan, Australia and New Zealand, where it is registered for protection of essentially every crop now under cultivation" (Dow AgroSciences, 2015). With 1700 tons sold per year in Italy, by itself or in combination with other aa.ii., in 115 authorised phytosanitary products (Ministero del lavoro della salute e delle politiche sociali, 2014), it is one of the most used insecticides in the country, for both agriculture and domestic/urban use (ISPRA, 2015), thus the high frequency of its presence in pollen is hardly surprising. The Italian monitoring project BEENET, which ran from 2011 to 2014, also found that chlorpyrifos was a frequent contaminant of pollen across Italy, with cases of 100% positivity in Valle d'Aosta and Sicily (Ministero delle politiche agricole alimentari e forestali, 2015). Furthermore, chlorpyrifos was found in colonies with poisoning symptoms hit by sudden death in Puglia in 2012 (probably due to chlorpyrifos and dimethoate treatments on grapevines), and in the province of Bolzano in 2013 (probably due to post-blossoming chlorpyrifos treatments on apple trees) (Ministero delle politiche agricole alimentari e forestali, 2015). Residues of chlorpyrifos were also found with high frequency and high levels in samples of organic honey collected in Italy (Chiesa et al., 2016).

In the U.S., a survey of pesticide residues on hive matrices, found that chlorpyrifos was the third most prevalent and abundant pesticide detected in the hive (Mullin et al., 2010). DeGrandi-Hoffman et al. (2013) showed that sublethal exposure of chlorpyrifos (967 μg kg^{-1} in pollen, 310 μg kg^{-1} in bee bread, 81 μg kg^{-1} in bees nursing queens,

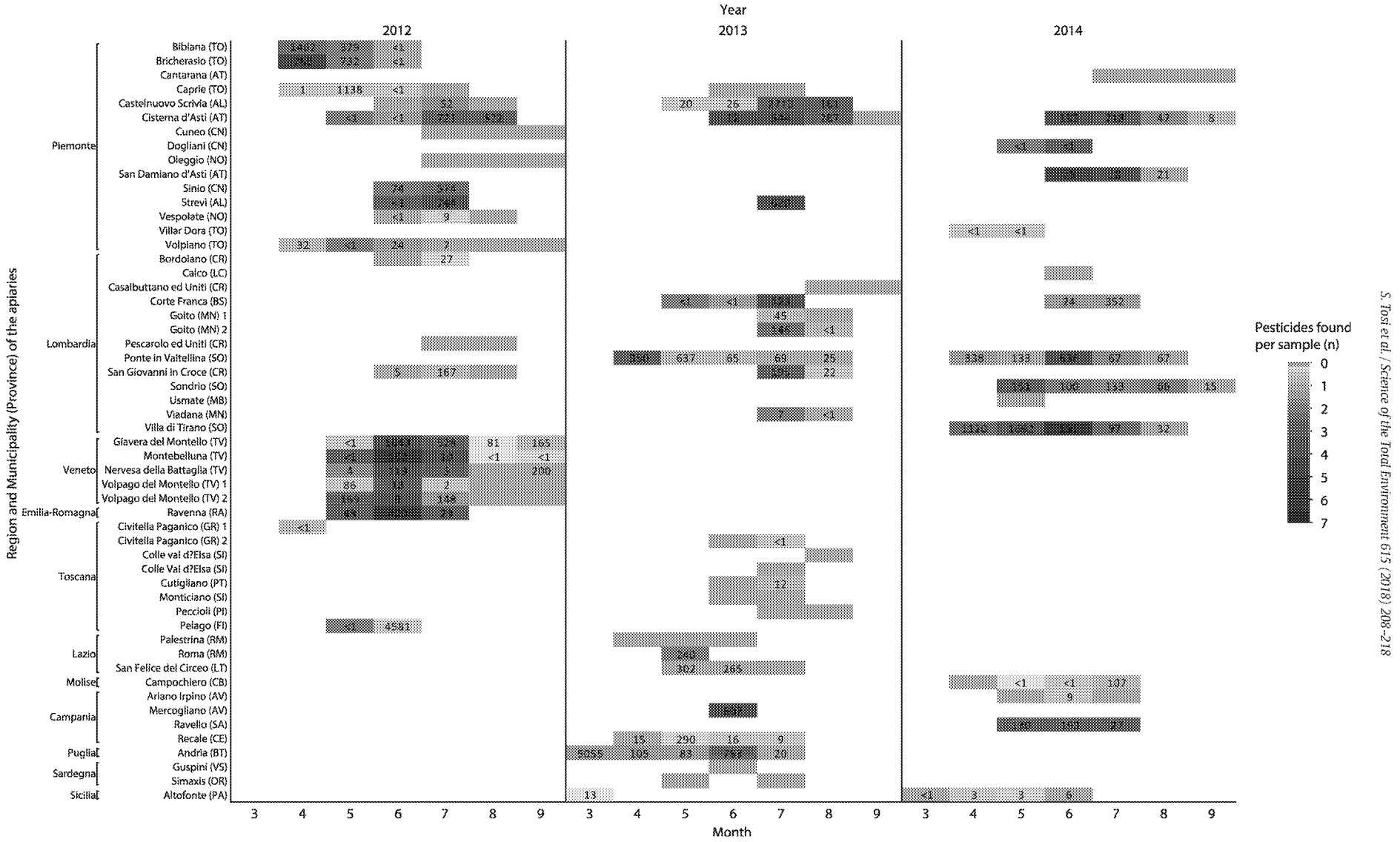

Fig. 2. Maximum number of pesticides and maximum Hazard Quotient (HQ) of single pollen samples in relation to apiary location (top: north; bottom: south and islands), year (2012–2014) and month (3–9, corresponding to March–September) of collection. We estimated the maximum Hazard Quotient (HQ) using the methods described by (Stoner and Eitzer, 2013; Traynor et al., 2016). The HQ was calculated dividing the maximum residue (μg kg^{-1}) of each pesticide by its respective oral LD50 (μg bee^{-1}) (EFSA, 2011). We estimated the HQ of pollen samples which typically contained multiple residues, assuming additive toxic effects. We excluded possible synergistic or antagonistic effects due to lack of quantitative data on interactions between most of the pesticides under assessment. The HQ is reported when at least one sample was contaminated. Darker colors reflect the increased number of pesticides (green = 0 pesticides/sample, darker red = 7 pesticides/sample). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Positive pollen samples across years (2012–2014) and months (3–9, corresponding to March–September). We also report the percentage of positive samples containing insecticides or fungicides and the frequency of systemic pesticides (which may be either insecticides or fungicides). We only show the positive results (>0%).

Year	Month	N	Positive samples (%)	N of detected a.i. (% positive)								A.i. type (% positive)		
				1	2	3	4	5	6	7	>1	Insect.	Fung.	Syst.
2012	4	20	60	25	20	5	5	5			35	88	12	28
	5	37	73	14	35	16	5	3			59	40	60	27
	6	56	86	23	16	13	13	11	9	2	63	24	76	39
	7	60	60	17	15	12	10	5	0	2	43	46	54	36
	8	39	8	5		3					3	60	40	80
	9	26	31	31								63	38	50
	Total	238	56	18	15	9	7	5	2	1	38	39	61	36
2013	3	6	50	33	17						17	75	25	50
	4	6	67	33	17	17					33	86	14	43
	5	22	68	50	18						18	84	16	37
	6	41	63	24	15	7	7	5	2	2	39	45	55	42
	7	50	62	28	8	12	8	2	4		34	59	41	37
	8	23	35	22	4	9					13	46	54	38
	9	4	0											
	Total	152	57	29	11	8	5	2	2	1	28	57	43	40
2014	3	2	100	100							0	100	0	0
	4	24	58	42	8	8					17	80	20	35
	5	33	64	21	3	24	15				42	36	64	38
	6	46	85	20	17	24	9	9	7		65	29	70	38
	7	36	81	25	25	19		11			56	38	65	32
	8	16	75	38	25	13					38	70	30	15
	9	7	43	29	14						14	75	25	25
	Total	164	73	27	15	18	5	5	2		46	40	60	34
Overall	3	8	63	50	13						13	83	17	33
	4	50	60	34	14	8	2	2			26	85	15	33
	5	92	68	25	20	15	8	1			43	45	55	33
	6	142	79	23	15	15	10	8	6	1	56	30	69	39
	7	147	66	22	16	14	7	5	1	1	44	48	53	35
	8	78	29	17	6	6					13	61	39	32
	9	37	30	27	3						3	67	33	42
	Total	554	62	24	14	12	6	4	2	1	38	44	56	36

in average) reduced queen emergence, possibly due to compromised immunity in developing queens. Urlacher et al. (2016) found that bees fed with doses (~0.05 ng bee^{-1}) ~5000 times lower than its LD_{50} (250 ng bee^{-1}(Lewis et al., 2016)) had slower appetitive learning ability, and a reduced specificity of memory recall. Seventeen percent of our pollen samples contained doses of chlorpyrifos higher than 0.05 ng bee^{-1} (corresponding to 4.17 μg kg^{-1}, based on the maximum daily pollen consumption of a bee, 12 mg/bee/day, EFSA, 2012), and could therefore elicit sublethal effects on bees. Learning and memory are of utmost importance for the behaviour of foraging bees, and their impairment may result in negative consequences for colony health and survival (Henry et al., 2015, 2012). The high proportion of samples containing chlorpyrifos found in this study, combined with the relatively high average level of residues and Hazard Quotient are of great concern for the health status of honey bees and other pollinators, especially considering that the use of chlorpyrifos is globally widespread.

The risks posed by chlorpyrifos on human health, especially on child neural development, caused the United States Environmental Protection Agency (US EPA) to ban its use as a household pesticide (US EPA, 2011a, 2011b). In October 2015, strong of further evidence on the risk to human health, EPA proposed to revoke all food residue tolerances for the insecticide chlorpyrifos (US EPA, 2015), which would signify a ban on all agricultural uses. In Europe, in 2014, the European Food Safety Agency (EFSA) published an updated toxicological risk assessment of chlorpyrifos for humans, in which, in the light of the new available data, the reference toxicological values were decreased (EFSA, 2014). EFSA also highlighted the risks of exceedance of the Acute Reference Dose deriving from the current agricultural use. Our study confirms the results of the EFSA modelling: 99% and 52% of the samples with residues of chlorpyrifos exceeded the new ADI (1 μg kg^{-1}) and ARfD (5 μg kg^{-1}) safety values, respectively.

Over the 3 years, 13% of the monitored apiaries were exposed to residue levels considered of concern for bee health (Hazard Quotient higher than 1000). Four insecticides had the highest maximum HQ, the neonicotinoids imidacloprid and thiamethoxam, and the organophosphates dimethoate and chlorpyrifos (Table 2, Fig. 2). The wide and diverse contamination observed for the different apiaries shows that specific risk levels apply to each location (Fig. 2), likely depending on local forage availability, mode of pesticide application, and specific microclimatic conditions. This explains the difficulty in generalising conclusions on the impact of pesticides on honey bee health.

The use of three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) was restricted for certain uses by the Italian government in 2009 and by the EU in 2013, as a consequence of their side-effects on honey bee health. The EU restriction prohibited the use of imidacloprid, clothianidin, and thiamethoxam in seed treatments, soil treatments, or spray treatments before and during flowering, on crops attractive to bees (uses in greenhouses are allowed) (EU, 2013). However, 2 of the 3 neonicotinoids screened in our study (imidacloprid and thiamethoxam) were detected in pollen samples within all 3 sampling years (17% of neonicotinoids positive samples overall, see Table 2 and Supplementary Table S1), with high maximum HQ levels. Imidacloprid concentrations were higher than the limit of concern (HQ > 1000, see Material and methods) in 12% of its positive samples. Therefore, despite the actual neonicotinoid use restriction, honey bees are still exposed to alarmingly high levels of these pesticides. Possibly, bees foraged on wild flowers blooming in proximity of crops that were non attractive to bees, and therefore legally sprayed with neonicotinoids. In fact, the inclusion of flowering strips, buffer zones, and cover/catch crops as good farming practices can increase the exposure of bees through drift of pesticides out of the treated fields (David et al., 2016; Simon-Delso et al., 2017). Furthermore, pesticides, especially systemic ones, may be found in environmental reservoirs, such as soil and water, thus providing multiple routes for exposure of wildlife (Navarro et al., 2007; Samson-Robert et al., 2014). Some systemic pesticides such as neonicotinoids (i.e. imidacloprid, thiamethoxam) are also particularly persistent in soil

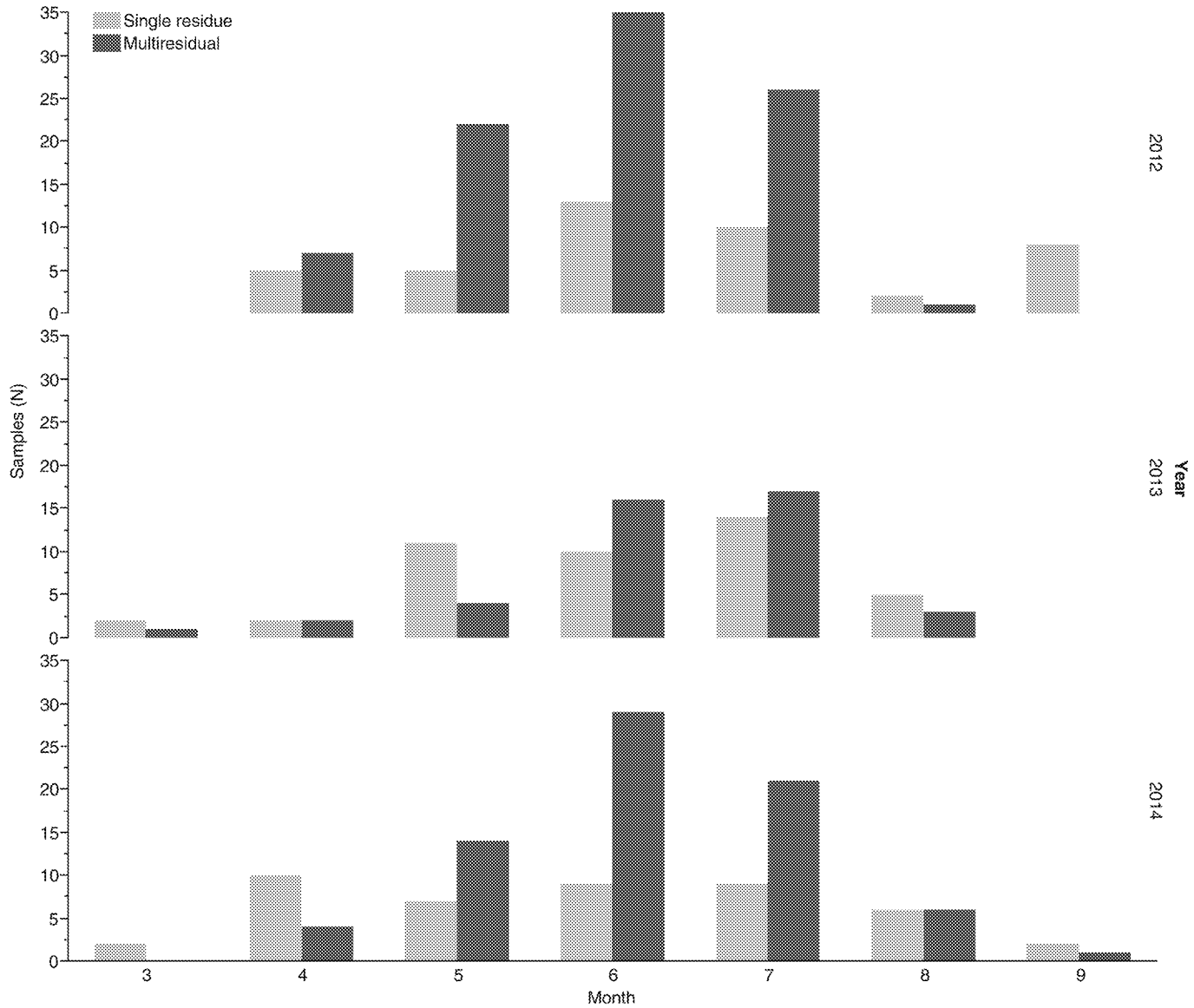

Fig. 3. Number of single-residue (light grey) and multiresidual (dark grey) pollen samples in relation to year (2012–2014) and month (3–9, corresponding to March–September) of collection by the bees. Sample size and further details are reported in Table 3.

(Botías et al., 2015; Goulson, 2013; Jones et al., 2014). As a consequence, the plants could take up the pesticide years after the actual treatment, resulting in prolonged contamination across years.

All apiaries in this study were located in agricultural areas, thus increasing the likelihood of occurrence of plant pest control treatments containing the detected pesticides (see Supplementary Table S2 for details on pesticides use). More than half of the source apiaries were managed according to the organic production rules set down in the EU regulation N.889/2008 (European Commission, 2008), which prescribes that "the siting of the apiaries shall be such that, within a radius of 3 km from the apiary site, nectar and pollen sources consist essentially of organically produced crops and/or spontaneous vegetation and/or crops treated with low environmental impact methods. […] The above mentioned requirements do not apply where flowering is not taking place, or the hives are dormant". Thus, organic beehives may be placed next to non-organically produced crops that are treated with pesticides because they are either not considered as nectar and pollen sources for bees (i.e. grapevines, olive trees), treated according to low impact methods, or not flowering. Pesticides can thus be applied to the crops around organic beehives in numerous occasions, and our study shows that this happens routinely, exposing bees to pesticides e.g. through drift on spontaneous plants. Our results thus question the current EU legislation concerning organic beekeeping. The contamination of organic colonies by agricultural pesticides was likewise highlighted by Chiesa et al. (2016), who found presence of pesticide residues in organic honey samples. However, some pesticides that we detected could also be used as veterinary applications (i.e. to treat livestock), indicating this as another possible cause of contamination.

Fungicides were considered for a long time as being safe for honey bees (Elston et al., 2013; Everich et al., 2009; Malone et al., 2007), and are even sprayed on crops during blossoming. However, various studies have shown that the impact of fungicides on bee health is not harmless. For example, high pollen contamination by fungicides was related to the phenomenon defined "entombed pollen", which was associated with honey bee mortality (Chapman et al., 2006). Pettis et al. (2013) found that the consumption of pollen collected from US crops, with high levels

of residues of fungicides, increased the bees' likelihood of being infected with the gut parasite *Nosema ceranae*. In Europe, a monitoring study based on observation of 330 colonies, found a significant correlation between colony disorders (dead, weak or queenless) and the presence of fungicide residues in the colony (Simon-Delso et al., 2014). Other studies highlighted the presence of adverse synergistic effects on honey bee health between fungicides, insecticides, and acaricides (Sanchez-Bayo and Goka, 2014; Sgolastra et al., 2017a; Zhu et al., 2014), including thermoregulation impairment (Vandame and Belzunces, 1998) and reduced repellency to pyrethroids (Thompson and Wilkins, 2003). DeGrandi-Hoffman et al. (2013) found that the sublethal effects of chlorpyrifos were greater when a fungicide which affects respiration (Pristine® BASF, Research Triangle Park, NC, USA, containing the aa.ii. boscalid and pyraclostrobin) was added to the pollen fed to the experimental colonies. A key role in the interactive effects elicited by pesticides mixtures is played by those fungicides that inhibit the detoxicative cytochrome P450 monooxygenase activity, such as the DeMethylation Inhibitor (DMI) tebuconazole (Johnson et al., 2013), that was found with frequencies over 10% in our study, in ten cases together with imidacloprid residues, and in one case together with both imidacloprid and thiamethoxam. Fungicides also potentially disrupt honey bee mycoflora, which is essential to process the pollen that will be stored in the hive as bee bread (Yoder et al., 2013). Thus, the high level of fungicides found in our study (56% of positive samples overall, Table 3) is cause for concern, also because we show that in field conditions bees can be simultaneously exposed to combinations of pesticides that elicit synergistic effects, which may lead to severe negative effects on their health.

Monitoring residues in corbicular pollen, as compared to beebread, reduces the possibility of underestimating pesticide levels due to pesticide degradation over time, and provides more precise information of the actual period of exposure. In fact, the beebread can be stored in the hive for months after collection in the field, while our corbicular pollen was sampled at maximum 7 days after collection. Also, because we sampled the pollen before its introduction into the hive, we can assume that the detected residues originate from applications on agricultural crops and not from treatments within the hive. Thus, we desume that the presence of chlorfenvinphos residues was not due to the use for control of *Varroa destructor* (Boi et al., 2016; Serra-Bonvehi and Orantes-Bermejo, 2010) but to illegal applications on agricultural crops. Our results highlighted that pesticides were present in the bee-collected pollen throughout the active beekeeping seasons (Fig. 3 and Table 3). Persistent sublethal pollen contamination may cause extended periods of immunosuppression among immature and adult bees, opening the way to viruses and other pathogens (DeGrandi-Hoffman et al., 2013). The prolonged and concurrent contamination by multiple pesticides of different chemical groups, evidenced in our study, could lead to adverse chronic and synergistic effects (Gill et al., 2012; Sanchez-Bayo and Goka, 2014; Sgolastra et al., 2017a; Zhu et al., 2014).

The results of our survey raise concern on the side-effects on human health, considering that we found residues of both illegal pesticides and illegal concentrations of authorised pesticides (39% of residues exceeded the EU Acute Reference Dose, the Acceptable Daily Intake and/or the Maximum Residue Limit). Although there are no official data on the market of pollen as a food supplement, apicultural experts report that production of pollen is increasing to satisfy a growing consumer demand (an Internet search for "pollen" in Italian will yield hundreds of websites citing the beneficial properties of bee-pollen consumption). Of the beekeepers involved in this study, 50% were collecting pollen for human consumption (personal or commercial), while another 20% were planning to start commercial pollen collection but gave up due to the results of this study, which show that pollen can harbour levels of pesticides not considered acceptable for human health, instead of being a natural "superfood". Our results show that the current agricultural prescriptions are not sufficient to ensure the safety of bee matrices, and consequently cause economic damage to beekeepers, especially those managing their hives according to the organic methods.

5. Conclusions

This 3-year monitoring survey showed a widespread and prolonged pollen contamination by multiple insecticides and fungicides under the current agricultural pesticide application practices, strengthening the evidence that managed and wild pollinators in rural areas, even those supposedly managed according to low environmental impact methods, are routinely exposed to multiple pesticides (Botías et al., 2017; David et al., 2016; Hladik et al., 2016; Lambert et al., 2013; Long and Krupke, 2016; Mullin et al., 2010; Pettis et al., 2013). The frequently high values of Hazard Quotient, Acute Reference Dose, Acceptable Daily Intake, and Maximum Residue Limit show that the pesticide contamination levels in the environment are cause for concern for bee, human, and environmental health. Our results also suggest that pesticide risk assessment procedures should investigate field-realistic exposure to pesticide combinations. Finally, we demonstrate that bee-collected pollen monitoring is a valuable tool for environmental monitoring of pesticide contamination, including the detection of illegal uses.

The residues of pesticides detected in each of the 3 years of the survey are reported in Supplementary Table S1, and more details on their use is reported in Supplementary Table S2. Supplementary Fig. S1 reports positive and negative samples (N) per region.

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.scitotenv.2017.09.226.

Acknowledgments

We are grateful to the beekeepers that participated to the survey on a voluntary basis. We thank the National Union of Italian Beekeepers (Unaapi), and the apiary technicians for their contribution to data collection. We thank Edoardo Carnesecchi for the feedback on the manuscript, and Serena Magagnoli for the bee photo

Funding

This work was funded by the Italian Minister of Forestry and Agricultural Policies (MiPaaF, D.M. 10197, 6 May 2010). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Conflicts of interest

The authors declare no competing interests.

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